

Overestimation of Organic Phosphorus in Wetland Soils by Alkaline Extraction and Molybdate Colorimetry

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Accurate information on the chemical nature of soil phosphorus is essential for understanding its bioavailability and fate in wetland ecosystems. Solution phosphorus-31 nuclear magnetic resonance (³¹P NMR) spectroscopy was used to assess the conventional colorimetric procedure for phosphorus speciation in alkaline extracts of organic soils from the Florida Everglades. Molybdate colorimetry markedly overestimated organic phosphorus by between 30 and 54% compared to NMR spectroscopy. This was due in large part to the association of inorganic phosphate with organic matter, although the error was exacerbated in some samples by the presence of pyrophosphate, an inorganic polyphosphate that is not detected by colorimetry. The results have important implications for our understanding of phosphorus biogeochemistry in wetlands and suggest that alkaline extraction and solution ³¹P NMR spectroscopy is the only accurate method for quantifying organic phosphorus in wetland soils.

Introduction

The various phosphorus compounds present in the natural environment exhibit marked differences in their behavior and bioavailability (1). Information on the chemical nature of phosphorus is, therefore, the basis for understanding its biogeochemistry and long-term fate in ecosystems. Organic phosphorus is of particular significance, and there is renewed interest in its dynamics in the environment (2). In wetlands it constitutes a major component of the soil phosphorus (3, 4) and plays a key role in the nutrition of organisms (5). The accurate determination of soil organic phosphorus is, therefore, essential for understanding the biogeochemistry of wetland ecosystems.

There is currently no way to determine soil organic phosphorus directly. It can be estimated following ignition at high temperature (6), although this is inappropriate for organic soils due to volatilization losses and incomplete

recovery of inorganic phosphate by acid extraction prior to ignition (7, 8). Soil organic phosphorus is, therefore, more accurately determined in organic soils by extraction (9). Prior to the application of NMR spectroscopy to soil phosphorus in 1980 (10), soil organic phosphorus was commonly determined by alkaline extraction and molybdate colorimetry (9, 11). The method is still used widely and is integral to sequential extraction schemes that fractionate soil phosphorus on the basis of chemical solubility (12–15).

The most common colorimetric detection procedure is based on the reaction of free phosphate with an acidified molybdate reagent to yield phosphomolybdate heteropolyacid; the complex is then reduced to a blue compound and determined spectrophotometrically (16). Total phosphorus is measured in the extract by some form of digestion, and the organic fraction is calculated as the difference between total phosphorus and inorganic phosphate. In sequential fractionation schemes, the inorganic phosphate extracted in strong alkali is assumed to originate from complexes with iron and aluminum, while the phosphorus that does not react with molybdate is classified exclusively as organic phosphorus (13–15).

The colorimetric detection procedure involves two potentially important sources of error (9). First, organic phosphorus is overestimated in the presence of inorganic polyphosphates, which do not react with molybdate and are, therefore, included in the organic phosphorus fraction (17). Second, humic acids are precipitated from alkaline extracts by acidification to prevent interference in color detection (18), but this introduces the possibility that phosphate is coprecipitated and not measured by subsequent molybdate reaction (19). These errors are usually assumed to be negligible, although neither has been investigated systematically for wetland soils.

The development of solution ³¹P NMR spectroscopy for application to alkaline soil extracts means it is now possible to quantify organic phosphorus without molybdate colorimetry (20). Here we report the use of this technique to assess the accuracy of inorganic and organic phosphorus determination by alkaline extraction and molybdate colorimetry in organic soils from the Florida Everglades.

Materials and Methods

Samples of benthic floc and underlying soil were taken during February 2004 from four sites in the Florida Everglades, selected to provide a series of distinct wetlands with a range of chemical properties. The sites were (i) a nutrient-enriched area of a hardwater marsh in Water Conservation Area 2A (termed F1 in previous publications) supporting a monospecific cattail (*Typha* spp.) community (21); (ii) a treatment wetland (Stormwater Treatment Area 1 West, Cell 4) constructed in 1994 on former agricultural land, supporting submerged aquatic vegetation, including coontail (*Ceratophyllum demersum* L.) and naiad (*Najas huadalupeensis* (Spreng.) Magnus) (22); (iii) an open-water slough in a pristine area of softwater marsh in Water Conservation Area 1, supporting emergent macrophytes and a periphyton community comprised of green algae and diatoms (23); and (iv) an open-water slough in an unenriched area of hardwater marsh in Water Conservation Area 2A (termed U3 in previous publications), dominated by calcareous periphyton mats comprised of calcium-precipitating cyanobacteria and diatoms (21, 24).

At each site, four cores (10-cm diameter) were taken to 10 cm depth in the organic soil layer. The cores were transported on ice to the laboratory (approximately 5 h),

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TABLE 1. Chemical Properties of Benthic Floc and Underlying Soil (0–10 cm) from Four Wetland Sites with Contrasting Chemistry in the Florida Everglades, U.S.A.

sample	pH	loss on ignition (%)	total elements (g kg ⁻¹ dry wt)						
			C	N	P	Al ^a	Ca	Fe	Mn
Cattail Marsh									
benthic floc	7.4	84	389	26.8	1.12	1.17	41.9	0.23	0.05
soil	7.6	90	426	30.9	0.27	1.58	22.8	0.21	0.02
Treatment Wetland									
benthic floc	8.0	48	274	16.0	0.79	1.70	82.8	0.62	0.26
soil	7.0	89	444	25.3	0.25	2.18	28.1	1.49	0.18
Softwater Slough									
benthic floc	5.8	90	440	37.8	0.31	2.18	10.0	<0.1	0.08
soil	5.6	96	468	37.7	0.23	1.97	9.7	0.30	0.05
Calcareous Slough									
benthic floc	7.9	36	222	14.2	0.21	0.66	64.7	0.15	0.44
soil	7.5	89	431	33.5	0.19	2.02	25.7	1.04	0.10

^a Values for aluminum were obtained from analysis of samples taken at the same locations eight months previously (29, 30).

where unconsolidated benthic floc was separated immediately from underlying soil. The floc consisted primarily of plant detritus and a smaller amount of algae, although there was a considerable mineral component in samples from the treatment wetland and the calcareous marsh. Material from the four replicate cores from each site was combined to produce single composite samples of floc and soil for each location. Recognizable plant matter (i.e., large stems and roots) and shells were removed by hand and the samples were stored at 4 °C.

Loss on ignition was determined by igniting samples at 550 °C for 3 h. Total phosphorus was determined in the ignited samples by acid digestion (6 M HCl) and automated molybdate colorimetry (25). Total carbon and nitrogen were determined by combustion and gas-chromatography using a Flash EA1112 CN analyzer (CE Elantech, Lakewood, New Jersey, U.S.A.). Soil pH was determined in a 1:20 ratio of dried soil to deionized water (1:2 on a wet weight basis). Total calcium, iron, and manganese were determined by digestion in concentrated sulfuric acid and selenium catalyst (26), with detection by inductively coupled plasma optical-emission spectrometry (ICP-OES). Results are reported on the basis of oven-dried material (105 °C, 24 h).

Phosphorus was extracted using a simplified version of the sequential fractionation procedure outlined by Ivanoff et al. (13). Fresh soil or benthic floc was extracted sequentially in 0.5 M NaHCO₃ (16 h), 1.0 M HCl (1 h), and 0.5 M NaOH (16 h) in a 1:50 soil-to-solution ratio on the basis of oven-dry soil. The acid extraction step removes inorganic phosphate and cations that can interfere with subsequent extraction of organic phosphorus in alkali. Each sample was extracted in triplicate at ambient laboratory temperature using a reciprocal shaker. The NaOH extracts were centrifuged (7000 g, 30 min), filtered (glass microfiber filter), and an aliquot taken for total phosphorus determination by ignition and acid digestion as described above.

Inorganic and organic phosphorus were determined in the NaOH extracts by two procedures: colorimetry and solution ³¹P NMR spectroscopy. For colorimetry, inorganic phosphate was determined by reaction with molybdate following the method of Murphy and Riley (16). Prior to analysis, humic acids were precipitated using the method of Tiessen and Moir (18) adapted for the stronger NaOH concentration used here. Briefly, extracts were acidified to pH 1.5 with 1.0 M H₂SO₄, chilled (4 °C, 30 min), and centrifuged (7000 g, 10 min). The supernatant was decanted and the pH adjusted to slightly acidic (approximately pH 5.0) using phenolphthalein indicator. Color development proceeded for 20 min after the addition of molybdate reagent, during which time no further precipitation was observed. Similar values were observed when parallel analyses were conducted on samples without precipitation of humic acids (i.e., color correction using a control sample with molybdate-free reagent only), but were considered unreliable due to continual precipitation during analysis. Organic phosphorus was calculated as the difference between total phosphorus and inorganic phosphate. All replicate extracts were analyzed separately, and results were corrected by subtracting the mean phosphorus concentration of three blank extracts.

For solution ³¹P NMR spectroscopy, equal volumes of the replicate NaOH extracts were combined, immediately frozen at -80 °C, and lyophilized. Each lyophilized extract (~100 mg) was redissolved in 0.1 mL of deuterium oxide and 0.9 mL of a solution containing 1.0 M NaOH and 0.1 M Na₂EDTA (ethylenediaminetetraacetate), and then transferred to a 5-mm NMR tube. The deuterium oxide provided an NMR signal lock and the NaOH raised the pH to > 13 to ensure consistent chemical shifts and optimum spectral

TABLE 2. Inorganic and Organic Phosphorus Determined by Molybdate Colorimetry and Solution ³¹P NMR Spectroscopy in NaOH Extracts of Benthic Floc and Underlying Soil (0–10 cm) from Wetlands in the Florida Everglades, U.S.A.^a

	NaOH total P ^b (mg P kg ⁻¹)	molybdate colorimetry ^c (% NaOH extractable P)		solution ³¹ P NMR spectroscopy (% NaOH extractable P) ^d			overestimation of organic P (%)
		phosphate	organic P	phosphate	organic P	pyrophosphate	
Cattail Marsh							
benthic floc	452 ± 29 (40)	9.6	90.4	31.1	66.2	2.7	37
soil	101 ± 7 (38)	6.4	93.6	33.6	66.4	ND	41
Treatment Wetland							
benthic floc	174 ± 14 (22)	7.9	92.1	33.0	67.0	ND	37
soil	129 ± 6 (52)	5.3	94.7	27.0	73.0	ND	30
Softwater Slough							
benthic floc	226 ± 33 (74)	11.4	88.6	22.2	57.6	20.2	54
soil	109 ± 4 (47)	6.0	94.0	27.3	67.2	5.5	40
Calcareous Slough							
benthic floc	24 ± 3 (12)	3.9	96.1	100.0	ND	ND	-
soil	78 ± 5 (40)	6.3	93.7	31.7	68.3	TR	37

^a ND, not detected; TR, trace (i.e., not quantifiable). ^b Values are the mean ± standard deviation of three replicate extracts, and the values in parentheses are the proportion (%) of the total phosphorus in the NaOH extract. ^c Determined following precipitation of humic material by acidification and chilling (18). Data are means of triplicate extracts with standard deviation <5% of the mean value. ^d Data are from triplicate extracts that were combined prior to NMR spectroscopy.

resolution. Solution ^{31}P NMR spectra were obtained on a Bruker Avance DRX 500 MHz spectrometer operating at 202.456 MHz for ^{31}P , using a $6\ \mu\text{s}$ pulse (45°), a delay time of 2.0 s, and an acquisition time of 0.4 s. Between 24 000 and 38 000 scans were acquired depending on the phosphorus concentration of the extract and broadband proton decoupling was used for all samples. Chemical shifts of signals were determined in parts per million (ppm) relative to an external standard of 85% H_3PO_4 and assigned to individual phosphorus compounds or functional groups based on literature reports (27). Signal areas were calculated by integration and spectra were plotted with a line broadening of 5 Hz. Data were corrected for inorganic phosphate in blank extracts.

The effect of precipitation of humic material and the pH of precipitation was investigated using benthic floc from the cattail marsh. The sample was lyophilized and triplicate samples extracted sequentially as described above. Humic acids were precipitated from NaOH extracts by acidifying aliquots (40 mL) to pH 0.2, 0.5, 1.0, or 1.5 using H_2SO_4 . The samples were diluted to 50 mL with deionized water, chilled (4°C , 30 min), and centrifuged (7000 g, 10 min). Unacidified samples were also analyzed. Aliquots of each sample were taken for total phosphorus analysis. Equal volumes of the three replicate extracts for each pH interval were then combined into a single sample, made alkaline (pH 13) with 5 M NaOH, and analyzed by NMR spectroscopy as described above.

Results

Chemical properties of the samples are reported in Table 1. The soils were all highly organic, with loss on ignition $>80\%$ for all except two calcareous samples of benthic floc. Soil pH was neutral or slightly alkaline for all samples except those from the softwater marsh. Total phosphorus concentrations in benthic floc ranged widely, being smallest in the calcareous marsh ($0.21\ \text{g P kg}^{-1}$) and largest in the enriched cattail marsh ($1.12\ \text{g P kg}^{-1}$). In soil, concentrations were low and relatively similar for all samples ($0.19\text{--}0.27\ \text{g P kg}^{-1}$).

Total phosphorus in NaOH extracts ranged between 24 and $452\ \text{mg P kg}^{-1}$, which represented between 12 and 74% of the total soil phosphorus (Table 2). The lowest values were for the two samples of calcareous benthic floc, in which most of the phosphorus was acid-extractable inorganic phosphate (28).

Estimation of inorganic and organic phosphorus in NaOH extracts by molybdate colorimetry was in serious error for all samples. Free phosphate determined by molybdate colorimetry was on average $7.1 \pm 2.4\%$ of the extracted phosphorus, with the remaining $92.9 \pm 2.4\%$, therefore, classified as organic phosphorus (Table 2). When extracts were analyzed by NMR spectroscopy, however, large proportions of inorganic phosphate were detected, as indicated by strong signals at 6.5 ppm in all spectra (Figure 1). This constituted, on average, $38.2 \pm 25.2\%$ of the extracted phosphorus (Table 2) and was the only compound detected by NMR spectroscopy in one case (benthic floc from the calcareous slough; Figure 1). However, this extract contained little phosphorus and exhibited poor spectral resolution, so the presence of organic phosphorus cannot be ruled out.

Where detected, organic phosphorus determined by NMR spectroscopy represented between 58 and 73% of the extracted phosphorus (Table 2). It consisted mainly of phosphate monoesters, with smaller concentrations of DNA (Figure 1). The sample of benthic floc from the treatment wetland also contained a small amount of phosphonate (4% of the organic phosphorus). This indicated the presence of

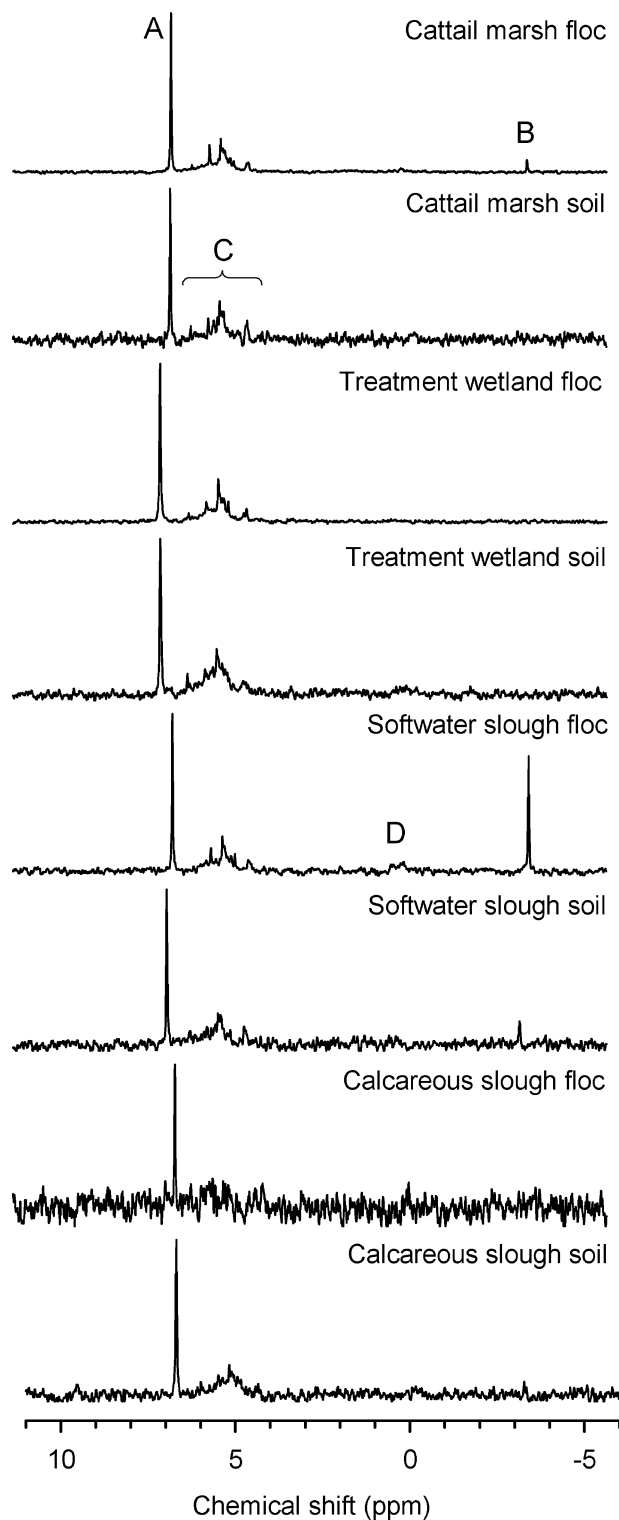


FIGURE 1. Solution ^{31}P NMR spectra of NaOH extracts of wetland soils from the Florida Everglades, U.S.A. The large signals at 6.5 ppm (A) indicate free inorganic phosphate, which is the form supposedly detected by molybdate colorimetry. Signals at -4 ppm (B) indicate inorganic pyrophosphate, which is not detected by colorimetry and is, therefore, included erroneously in the organic fraction. The remaining signals are from organic phosphates, with phosphate monoesters between 4 and 6 ppm (C) and DNA at 0 ppm (D).

the herbicide glyphosate (and its degradation product), which is used occasionally in the treatment wetland to control vegetation.

TABLE 3. Composition of Phosphorus Associated with Fulvic Acid (Acid Soluble) and Humic Acid (Acid Insoluble) in NaOH Extracts of Benthic Floc from an Enriched Cattail Marsh in the Florida Everglades, USA, Determined by Solution ³¹P NMR Spectroscopy

	total P (mg P kg ⁻¹)	recovery (%)	extracted P		
			phosphate (%)	organic P (%)	pyrophosphate (%)
no precipitation	478 ± 1.3		42	56	2
		Fulvic Acid (Acid Soluble)			
pH 1.5	218 ± 5.2	46	49	48	3
pH 1.0	216 ± 3.6	45	41	57	2
pH 0.5	214 ± 2.2	45	46	51	3
pH 0.2	216 ± 4.1	45	52	48	not detected
		Humic Acid (Acid Insoluble)			
pH 1.5	260 ± 5.4	54	37	62	1
pH 1.0	261 ± 3.9	55	44	55	1
pH 0.5	264 ± 2.5	55	39	60	1
pH 0.2	262 ± 4.3	55	35	62	3

Pyrophosphate was detected in quantifiable concentrations in only three samples, in which it represented between 3 and 20% of the extracted phosphorus (Table 2). A trace was detected in a fourth sample. Pyrophosphate does not react with molybdate, so is included erroneously in the organic phosphorus fraction when samples are analyzed by colorimetry. It therefore made a significant contribution to the overestimation of organic phosphorus by colorimetry in some samples. In total, molybdate colorimetry overestimated organic phosphorus by between 30 and 54%, excluding the extreme example in which only inorganic phosphate was detected by NMR spectroscopy (Table 2).

Approximately half the inorganic phosphate in the extract of benthic floc from the enriched wetland was coprecipitated with humic acids (soluble in alkali, insoluble in acid). There was little influence of precipitation pH between 0.2 and 1.5 on the amount ($p > 0.05$; one-way ANOVA) or composition of the phosphorus remaining in solution (Table 3). However, pyrophosphate was not detected when precipitation occurred at the most acidic pH (Figure 2), probably due to hydrolysis. Much of the phosphate remaining in solution following acidification did not react with molybdate, so it was presumably associated with fulvic acids (soluble in acid and alkali). Inorganic phosphate in this extract was, therefore, associated with humic and fulvic acids in similar proportions (Table 3).

Discussion

Alkaline extraction and molybdate colorimetry markedly overestimated organic phosphorus in a range of wetland soils from the Florida Everglades. This is a serious error in our understanding of wetland biogeochemistry, because organic phosphorus plays a key role in the nutrition of organisms in natural wetlands (5, 29) and the sequestration of pollutant phosphorus in artificial treatment wetlands (30). Previous studies in the northern Everglades used alkaline extraction and molybdate colorimetry to demonstrate the importance of soil organic phosphorus sequestration in the removal of pollutant phosphorus from the water column (3, 4). Our results suggest that these studies overestimated the role of organic phosphorus.

Inorganic phosphate in alkaline soil extracts is assumed to originate from complexes with both iron and aluminum (13, 14), although the latter appears to be most important in organic soils due to the reduction of iron oxides under anaerobic conditions (15, 19). Phosphate is strongly adsorbed to aluminum-peat complexes (31) and has been implicated in the retention of phosphate in wetlands. In particular, it was demonstrated that phosphate sorption in a wide range of wetland soils could be predicted solely from the concen-

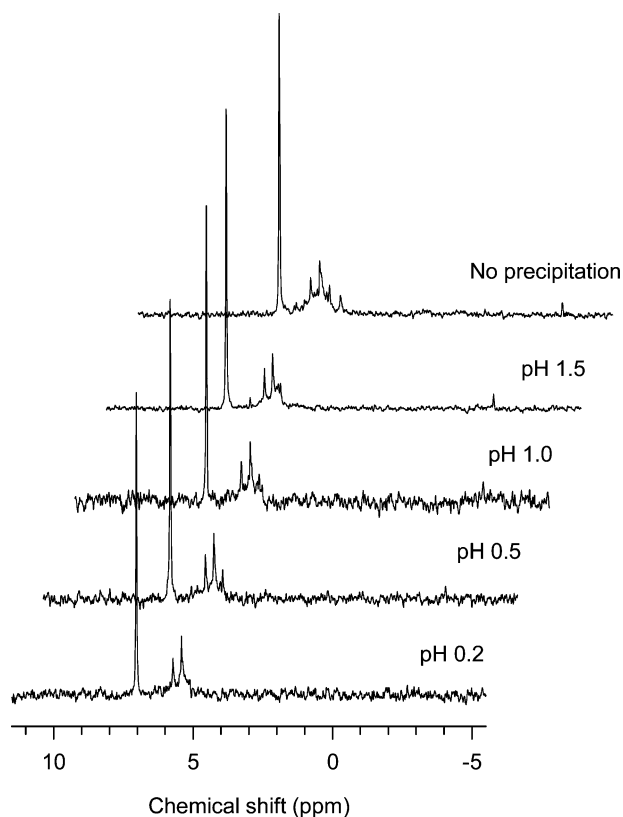


FIGURE 2. Solution ³¹P NMR spectroscopy of a NaOH extract of benthic floc from an enriched cattail marsh in the Florida Everglades following precipitation of humic acids by acidification to different pH values.

tration of amorphous (oxalate-extractable) aluminum (32). Given that inorganic phosphate is underestimated in strong alkaline extracts of organic soils by molybdate colorimetry, it seems likely that aluminum may be more important in regulating phosphate availability in wetlands than previously thought.

The additional inorganic phosphate detected by NMR spectroscopy was not an artifact of the analytical procedure, because organic phosphorus does not degrade to inorganic phosphate in alkaline solution (27). Molybdate colorimetry can underestimate inorganic phosphate when the reaction solution is too acidic (33), but this was ruled out by careful monitoring of pH during analysis. The results are in direct contrast to the conventional perception that molybdate

colorimetry underestimates organic phosphorus due to hydrolysis of acid-labile organic phosphates. However, studies involving the analysis of authentic organic phosphates suggest that such hydrolysis is negligible (12, 34).

The error in the colorimetric procedure was due in large part to the association of inorganic phosphate with organic matter. Both humic and fulvic acids were involved, although the nature of the association is unclear. The negative charge on phosphate and organic matter precludes direct sorption (9), although complexation can occur through metal bridges (19, 35–37). The formation of insoluble organic-metal-phosphate complexes can be considerable when alkaline extracts of mineral soils are acidified to pH 1.5–2.5, although the complexes begin to disintegrate as the solution becomes strongly acidic (19, 36, 38). In one study, this was responsible for an almost 20-fold decrease in the coprecipitation of phosphate with humic acids at pH 0.2 as compared to pH 2.5 (19). For organic soils, however, including those analyzed here, the coprecipitation of phosphate with humic acids is relatively uninfluenced by solution pH (19, 35), suggesting that metal complexation is of limited importance. An alternative mechanism, such as the occlusion of phosphate within organic structures, may therefore be responsible for the overestimation of organic phosphorus in wetland soils. Further experiments are required to investigate this.

Although error in the estimation of organic phosphorus by alkaline extraction and molybdate colorimetry is probably greatest for high organic matter soils, it seems evident from the literature that it may also occur during the analysis of soils from other ecosystems (9). For example, free phosphate was detected by solution ^{31}P NMR spectroscopy in alkaline soil extracts following dialysis (39) and in humic acids extracted from a wide range of soils (40–42). Discrepancies between colorimetric and spectroscopic measurements of phosphate were also reported for NaOH–EDTA extracts of temperate grassland soils with a wide range of organic carbon concentrations (43, 44).

The error could be minimized in extracts of mineral soils by precipitating organic matter at a strongly acidic pH (i.e., <0.5) prior to molybdate colorimetry, although this will not greatly influence results for organic soils (19). Ignition procedures can be used to estimate soil organic phosphorus, but are inaccurate for organic soils (7, 8), as exemplified by those studied here. Strong acid extraction clearly did not recover all inorganic phosphate, because a considerable proportion was extracted subsequently in strong alkali. This would be determined as organic phosphorus by the ignition procedure, resulting in a considerable overestimation. Given the error associated with procedures involving colorimetry or ignition, we conclude that alkaline extraction and solution ^{31}P NMR spectroscopy is the only reliable method for quantifying organic phosphorus in wetland soils (29, 30). In this respect, the single-step NaOH–EDTA extraction procedure may be most appropriate, because it precludes the hydrolysis of organic phosphorus that can occur during strong acid pretreatment (9, 29, 30).

In summary, conventional estimation of organic phosphorus by alkaline extraction and molybdate colorimetry substantially overestimated organic phosphorus in a series of wetland soils from the Florida Everglades. This was due mainly to the association of phosphate with organic matter, although the presence of pyrophosphate also contributed to the error in some samples. Given the widespread use of alkaline extraction and molybdate colorimetry to determine soil organic phosphorus, especially in sequential fractionation schemes, the results have important implications for our understanding of phosphorus biogeochemistry in wetlands. Future studies should directly quantify organic phosphorus in wetland soils by alkaline extraction and solution ^{31}P NMR spectroscopy.

Acknowledgments

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